Fetal Hemoglobin Production in Cultures of Primitive and Mature Human Erythroid Progenitors: Differentiation Affects the Quantity of Fetal Hemoglobin Produced per Fetal-Hemoglobin-Containing Cell

By George J. Dover, Teresa Chan, and Fritz Sieber

Single-cell microscopic immunodiffusion assays were used to determine the cellular mechanisms that regulate fetal hemoglobin (HbF) levels in cultures of primitive and late erythroid precursors obtained from human adult bone marrow. Two variables—the percentage of cells containing HbF (F cells) and the picograms (pg) of HbF/F cell—were assayed in cells derived from erythroid colony-forming units (CFU-E) and from erythroid burst-forming units (BFU-E) at 7 and 14 days in culture, respectively. The percentage of F cells among all nucleated cells from CFU-E-derived colonies (29.4% ± 12.5%, mean ± SD) was not significantly different (p = 0.2) from the percentage of F cells from BFU-E-derived bursts (37.3% ± 10.1%). Serial daily assays of all cells in cultures on days 3 through 7 and on day 14 revealed a marked increase in F cells between days 4 and 6 in culture. The average amount of HbF/F cell was less in CFU-E-derived F cells than in BFU-E-derived cells (3.5 ± 0.3 pg versus 6.2 ± 3.3 pg; p < 0.01), while adult hemoglobin (HbA) levels in CFU-E- and BFU-E-derived cells remained comparable (19.9 ± 2.2 pg versus 21.9 ± 5.3 pg, p = 0.3). These findings indicate that F cell number in culture is not significantly influenced by the relative maturity of the erythroid precursors from which the cells are derived. Differences in the levels of HbF between CFU-E- and BFU-E-derived cells are due to differences in the amount of HbF per F cell, not F cell number.

In normal adults, fetal hemoglobin (HbF) is confined to a small minority of red cells called F cells. HbF production may be increased in vivo by two independently regulated mechanisms: increased production of F cells, or increased production of HbF/F cell. Primitive erythropoietic progenitors, termed "erythroid burst-forming units" (BFU-E), isolated from either peripheral blood or bone marrow of normal adults produce more HbF than any other erythroid progenitors that develop in vivo. Cells derived from more mature erythroid precursors, termed "erythroid colony-forming units" (CFU-E), contain less HbF than cells derived from BFU-E under the same culture conditions.

The increased HbF production in cells derived from peripheral blood BFU-E is the result of increased production of F cells, and not an increase in the quantity of HbF/F cell. In this report, we wish to determine whether the differences in HbF production between CFU-E- and BFU-E-derived cells from normal bone marrow are due to differences in F cell number, differences in HbF/F cell, or both.

Materials and Methods

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Informed consent was obtained from normal, healthy adults according to the guidelines of The Johns Hopkins University School of Medicine and Hospital Joint Committee on Clinical Investigation.

Culture Conditions

Mononuclear cells from human bone marrow were cultured in methylcellulose as described by Iscove et al., except that Iscove's modified Dulbecco's medium (GIBCO, Grand Island, NY) was substituted for alpha-medium. In all cultures, 2 IU/ml of Step III sheep plasma erythropoietin (Connaught Laboratories, Willowdale, Ontario, Canada) was used.

Identification and Isolation of Erythroid Colonies

Erythroid colonies were identified in situ with an inverted microscope using a 415 ± 6 nm Soret band-pass filter (Infrared Industries, Waltham, MA). Erythroid colonies were classified as CFU-E-derived colonies or BFU-E-derived colonies (bursts) according to the following criteria. CFU-E-derived colonies, scored and harvested after 7 days in culture, were solitary, small, dense clusters consisting of 8-50 (typically about 15-20) cells that appeared dark (well hemoglobinized) when viewed through the Soret band filter. Bursts, scored and harvested after 14 days in culture, formed large, multifocal (≥3 subcolonies) colonies, and consisted of several hundred to several thousand well hemoglobinized cells. We were careful not to confuse CFU-E-derived colonies with less hemoglobinized bursts appearing at 7 days. In selected experiments, between days 7 and 14, we repeatedly examined CFU-E-derived colonies and less hemoglobinized colonies that were tentatively identified as early appearing bursts. CFU-E-derived colonies did not increase in size after 7 days in culture. However, colonies identified as bursts on day 7 in culture continued to proliferate and became progressively more hemoglobinized over the next 7 days. Merocyanine-540-mediated photosensitization of the mononuclear cell suspension inhibited the formation of CFU-E-derived colonies more effectively than the formation of bursts. Thus, the two types of colonies examined in this study were clearly the progeny of two different classes of progenitor cells: primitive progenitor cells (BFU-E) with a large proliferative potential, and more mature progenitors (CFU-E) with a limited prolifera-
Table 1. Percentage of Cells Containing HbF and HbA Peripheral Blood, 7-Day CFU-E-Derived Colonies, and 14-Day BFU-E-Derived Bursts From 5 Normal Subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Peripheral Blood (F/A Cell %)</th>
<th>Percent HbA Nucleated Cells</th>
<th>Percent HbF Nucleated Cells</th>
<th>F/A</th>
<th>Nucleated Cells</th>
<th>BFU-E Colonies†</th>
<th>F/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.9 ± 1.0§</td>
<td>71.0 ± 1.3</td>
<td>45.3 ± 7.8</td>
<td>63.8</td>
<td>75.3 ± 2.3</td>
<td>37.3 ± 1.2</td>
<td>49.5</td>
</tr>
<tr>
<td>2</td>
<td>0.2 ± 0.2</td>
<td>52.0 ± 2.0</td>
<td>26.0 ± 4.0</td>
<td>50.0</td>
<td>94.0 ± 2.0</td>
<td>33.3 ± 2.3</td>
<td>35.4</td>
</tr>
<tr>
<td>3</td>
<td>0.1 ± 0.2</td>
<td>74.7 ± 2.3</td>
<td>26.0 ± 2.0</td>
<td>34.8</td>
<td>88.0 ± 3.4</td>
<td>22.7 ± 2.3</td>
<td>25.8</td>
</tr>
<tr>
<td>4</td>
<td>4.0 ± 1.0</td>
<td>62.0 ± 2.0</td>
<td>37.3 ± 3.1</td>
<td>60.2</td>
<td>96.7 ± 1.2</td>
<td>45.3 ± 3.1</td>
<td>46.9</td>
</tr>
<tr>
<td>5</td>
<td>—</td>
<td>38.8 ± 2.2</td>
<td>12.4 ± 3.3</td>
<td>32.0</td>
<td>88.5 ± 9.5</td>
<td>34.6 ± 9.4</td>
<td>39.1</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>2.3 ± 2.0</td>
<td>59.7 ± 14.6</td>
<td>29.4 ± 12.5</td>
<td>48.2± 14.4</td>
<td>89.8 ± 8.8</td>
<td>37.3 ± 10.1</td>
<td>39.3 ± 9.5</td>
</tr>
</tbody>
</table>

*Mean number of colonies per 10⁵ nucleated cells for 5 subjects was 185 ± 39 (range 141–215).
†Mean number of bursts per 10⁶ nucleated cells for 5 subjects was 89 ± 25 (range 59–116).
‡F/A = (% HbF nucleated cells/HbA nucleated cells) x 100.
§Mean ± SD.

Single-Cell Assays

All single-cell assays were performed as previously described. Briefly, the cells were washed three times in buffered saline, cell aggregates were dispersed into single cells, and then divided into equal aliquots. Each aliquot was added to a mixture of agarose, New Methylene Blue, and antibody to either human HbF or adult hemoglobin (HbA). The agarose suspension was placed on glass slides, the cells were lysed with 0.2% Triton X-100, and the percentage of cells with HbF-anti-HbF or HbA-anti-HbA pericellular immunoprecipitate reactions were enumerated. Using microdensitometric methods, the amount of HbF or HbA in individual nucleated cells was quantitated. The lower detection thresholds of these antibodies for HbF and HbA are 3 and 14 pg, respectively. Nucleated cells without detectable HbA or HbF were found in all preparations. Since New Methylene Blue staining did not reliably discriminate those erythroid precursors with hemoglobin levels below our detection thresholds from monocyte or granulocyte precursors, the percentages of HbF- or HbA-bearing cells were expressed as a percentage of all nucleated cells (1,000–3,000 cells/assay). All probability estimates in this article were performed using the Mann-Whitney method when comparing samples with unequal variances, and the Student's t test when comparing samples with equal variances.

RESULTS

Assays of CFU-E- and BFU-E-Derived Cells

In 5 normal subjects (Table 1), the percentage of all nucleated cells containing HbF from CFU-E (29.4% ± 12.5%, mean ± SD) was not significantly different (p = 0.02) from that found in BFU-E-derived cells (37.3% ± 10.1%). It is important to note that these percentages represent minimal estimates, since the total number of cells from which the percent HbA cells and the percent HbF cells were derived contained some nucleated cells that were not erythroid in nature (see Materials and Methods). Contamination with nonerythroid cells was more evident in CFU-E than BFU-E assays, because CFU-E colonies, by virtue of their smaller size, were more difficult to remove without adjacent cells.

A more accurate estimate of the percentage of erythroid cells that contains HbF in cells derived from CFU-E and BFU-E is obtained by dividing the percentage HbF cells by the percentage HbA cells (F/A ratio). The validity of this approach rests on the previous observation that all F cells in culture contain detectable HbA. F/A ratios in CFU-E- and BFU-E-derived colonies, while not significantly different (p = 0.08), were actually greater in CFU-E-derived colonies (Table 1).

Serial Assays of Cells in Culture

The only erythroid cells detectable by Soret band analysis were small hemoglobinized colonies, typically containing 4–8 cells, each first observed between 3 and 6 days in culture. Since these small colonies, present in the early days of culture, are difficult to remove individually, we chose to assay all cells present on any
one day on days 3 through 7 and on day 14 and in two normal subjects. As seen in Fig. 1, the percentage of total cells containing HbA remained approximately the same between days 3 and 7, since in the culture conditions used, nonerythroid cells continued to divide during this time. The percentage of total cells in culture containing HbF and the F/A ratio increased dramatically between days 3 and 6 (Fig. 1). Comparable results were obtained in daily serial assays performed on an additional normal subject (data not shown). In this individual, peak F cell production (F/A = 44.8%) was obtained by day 5 and no significant increase in F cell production occurred thereafter.

**Picograms of HbF and Picograms of HbA in CFU-E- and BFU-E-Derived Cells**

The amount of pericellular immunoprecipitate in our assays is correlated directly with the picograms of HbF or HbA per cell. Anti-HbF assays of BFU-E-derived F cells from all subjects were associated with uniformly large immunoprecipitate reactions, while CFU-E-derived F cells had barely visible immunoprecipitates. Microdensitometry of cells from subject no. 5 showed that the mean amount of HbF in CFU-E-derived cells was 3.5 ± 0.3 pg (mean ± SD) of HbF/F cell, while BFU-E-derived F cells contained 6.2 ± 3.3 pg HbF/F cell (p < 0.01). Analysis of the relative distribution of HbF/F cell in BFU-E-derived F cells revealed no evidence of a distinct subpopulation of CFU-E-derived F cells. While 95% of CFU-E-derived F cells contained less than 4.0 pg HbF/F cell, BFU-E-derived F cells were normally distributed between 3.2 and 10.5 pg HbF/F cell. Only 10% of BFU-E-derived F cells had less than 4.0 pg of HbF. With anti-HbA antibody, immunoprecipitate reactions from BFU-E- and CFU-E-derived cells were indistinguishable. The average amounts of HbA per cell in subject nos. 5 were no different between CFU-E- and BFU-E-derived cells: 19.9 ± 2.2 pg versus 21.9 ± 5.34 pg (p = 0.3), respectively. These levels of HbF and HbA seen in cells from bone marrow derived BFU-E were similar to the levels previously determined for peripheral blood derived BFU-E (4.1–5.7 pg HbF; 21.8–35.1 pg HbA) and are comparable to microdensitometric determinations of HbF and HbA in erythroblasts from normal marrow (3.9–5.0 pg HbF; 20.9–24.6 pg HbA).

**DISCUSSION**

Our present data indicate that F cell production increases markedly between days 3 and 6 in culture (Fig. 1). Thereafter, comparable levels of F cells are seen at days 7 and 14 when CFU-E-derived colonies or BFU-E-derived bursts are assayed (Table 1). The major difference in CFU-E- and BFU-E-derived cells in relation to HbF production is the almost twofold difference in the quantity of HbF/F cell. Our observation that CFU-E-derived colonies and BFU-E-derived bursts contain comparable numbers of F cells suggest that in vitro conditions modulate HbF expression in early and late erythroid precursors. In support of this observation are recent reports that adult CFU-E-derived colonies and BFU-E-derived bursts produce equally elevated levels of HbF. Previous observations by other laboratories that CFU-E-derived bursts contain little or no detectable HbF indicate that culture conditions between laboratories differ subtly or that different assays for HbF have different lower detection thresholds.

These observations reemphasize the importance of analyzing alterations in HbF production at the cellular level. We know that HbF production in vivo is controlled by two separate variables. The first variable, the number of F cells produced, is inherited in normal individuals and in those individuals with sickle cell disease. In sickle cell disease, F cell production is determined by a genetic locus linked to the beta-globin gene and termed the F cell production (FCP) locus. The amount of HbF/F cell is a second variable whose expression seems to be independent of the number of F cells produced. At present, little is known about the factors, either genetic or environmental, that influence this second variable. What we do know is that HbF production in normal individuals is completed prior to the formation of reticulocytes, while HbA production continues throughout erythroid maturation. In at least some individuals with sickle cell disease, production of HbF continues through the reticulocyte stage so that the level of HbF/F cell finally attained may increase beyond the normal range.

In culture, these two variables, F cell number and HbF/F cell, also operate independently. Bursts derived from peripheral blood BFU-E of a single donor contain between 5% and 95% F cells/burst. However, the percentage of F cells produced per burst does not correlate with the average amount of HbF/F cell in these bursts.

Our observations on the cellular mechanisms that control HbF expression in vivo and in culture bear directly on a series of hypotheses set forth by Papayannopoulou and Stamatoyannopoulos. First, these investigators suggest that the potential to express HbF is progressively lost during normal erythroid differentiation. A corollary to this hypothesis is the presumption that HbF expression is the result of premature commitment to terminal differentiation by BFU-E that still have an active HbF program. Our
data indicate that the decreased potential for HbF production in CFU-E versus BFU-E-derived cells is due to a decreased capacity of CFU-E to produce F cells with high levels of HbF/F cell and not due to an inability of CFU-E to produce F cells. Second, on the basis of the similarity between increased HbF expression in BFU-E and increased in vivo F cell production during marrow expansion, Papayannopoulou suggested that the rise in F cell number during marrow expansion is due to premature terminal differentiation of BFU-E.9 Our observation that in vitro conditions can increase F cell number in CFU-E-derived cells suggests that increased F cell production may result from progeny of BFU-E or CFU-E. The prediction on the basis of information developed here would be that BFU-E-derived F cells would have higher levels of HbF/F cell than CFU-E-derived F cells. Third, Stamatoyannopoulos et al. have recently suggested that premature differentiation is a stochastic event that may be altered by genetic or environmental influences.20 The stochastic model is based on the presumptions that there exists a probability, p, which determines whether a BFU-E precursor will produce subcolonies all containing HbF, and a probability, 1 – p, that a BFU-E will produce subcolonies where no HbF is present. However, several laboratories using single-cell assays,23 isoelectric focusing,21 and polyacrylamide gel electrophoresis,22 have demonstrated the presence of HbF in all bursts. Under in vitro conditions where all bursts contain HbF, then 1 – p approaches 0 and the proof of a stochastic model becomes impossible. Our data might help to explain how such a stochastic model might indeed be compatible with HbF in all bursts. Since each individual burst contains numerous CFU-E-derived colonies, in vitro conditions that augment HbF production at both the BFU-E and CFU-E stage of differentiation are more likely to result in cultures where HbF is present in each burst.

In summary, the concept that the HbF program is gradually lost during differentiation remains attractive. However, this gradual loss of HbF expression and its reemergence is complex when analyzed at the cellular level. The observation that differentiation may alter HbF/F cell and apparently not F cell number may potentially lead to new insights into the mechanisms that control intracellular HbF levels.

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